REMARKS

Claims 76-91 were pending in the subject application. Claims 82-91 were withdrawn from consideration by the Examiner as directed to a non-elected invention. By this amendment, applicants have canceled Claims 82-91 without disclaimer or prejudice, and have amended Claims 76 and 79. The amendments place the application in condition for allowance or in better form for appeal.

Applicants maintain that the amendments do not raise an issue of new matter. Support for the amendments to Claim 76 and 79 can be found in the application at least on page 1, lines 13-14. Entry of the amendments is respectfully requested.

Upon entry of this amendment, Claims 76-81 will be pending and under examination.

Objections to the Claims

The Examiner objected to the claims because Claims 76 and 79 do not provide the full name for "*PKD2*." Applicants have hereinabove amended claims 76 and 79 to recite "polycystic kidney disease type 2 (*PKD2*)." Accordingly, reconsideration and withdrawal of this objection are respectfully requested.

Submission of Information Disclosure Statement Reference

Applicants attach hereto a legible copy of San Millan et al. (Am. J. Hum. Genet. 56: 248-253, 1995) as requested by the Examiner.

Applicants note that the San Millan et al. 1995 reference was listed in an Information Disclosure Statement submitted in connection with the subject application on March 24, 2003 and that the same reference was previously cited by the Patent Office during the prosecution of parent U.S. Patent Application No. 09/385,752, now U.S. Patent No. 6,228,591, from which the subject application claims benefit under 35 U.S.C.

§120. Accordingly, pursuant to 37 C.F.R. §1.98(d), a copy of this previously cited reference was not included with the March 24, 2003 Information Disclosure Statement.

Applicants request that the Examiner return a copy of previously submitted form PTO/SB/08B signed by the Examiner to indicate that the San Millan et al. reference listed on the form has been considered in the subject application.

CONCLUSIONS

No fee is deemed necessary in connection with the filing of this reply. However, if any fee is required in connection with this reply or to preserve the pendency of the subject application, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 01-1785.

Respectfully submitted,

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Dated: January 18, 2005
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By _____
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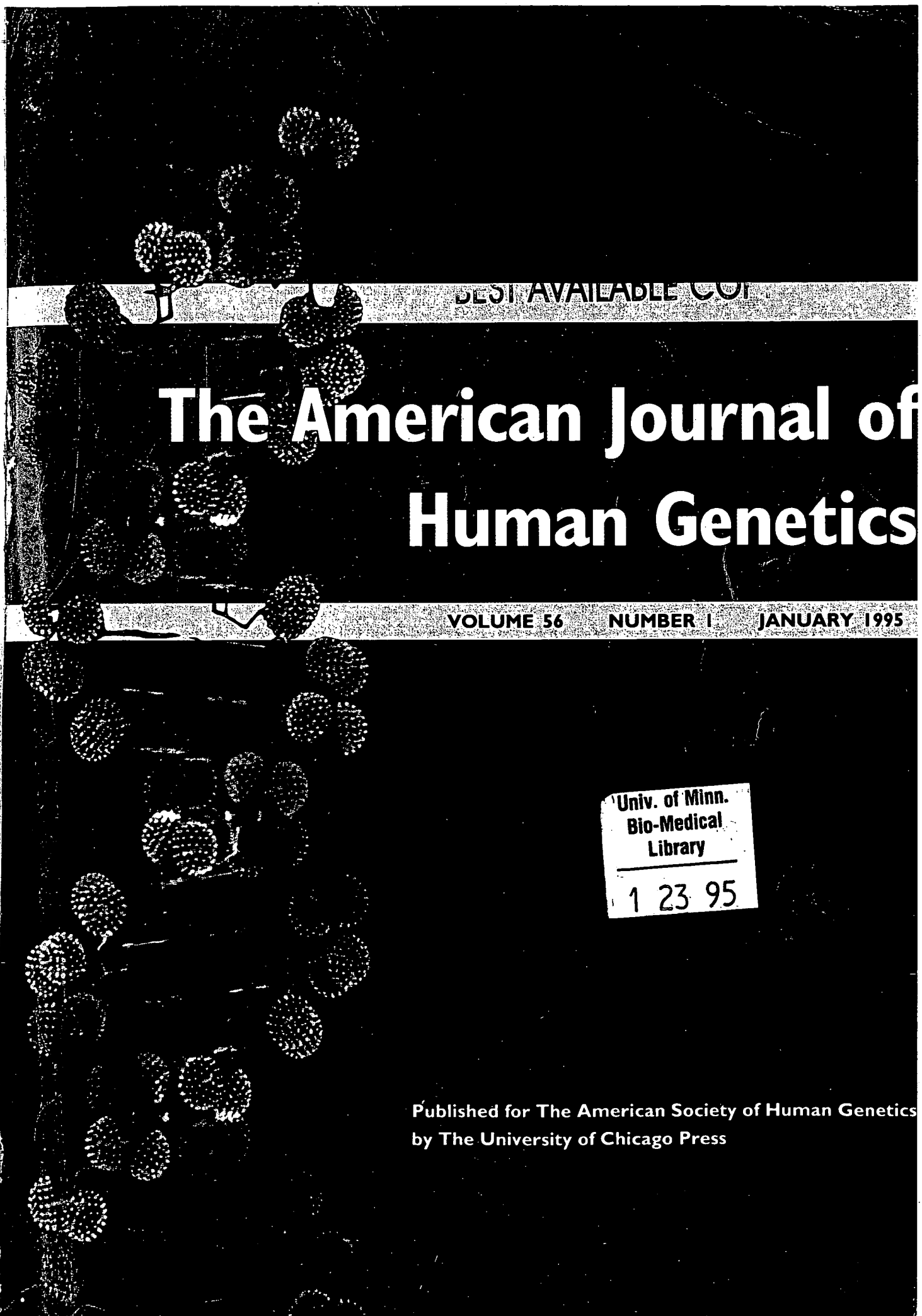

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Refining the Localization of the PKD2 Locus on Chromosome 4q by Linkage Analysis in Spanish Families with Autosomal Dominant Polycystic Kidney Disease Type 2

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Summary

Autosomal dominant polycystic kidney disease (ADPKD) is a genetically heterogeneous disorder. At least two distinct forms of ADPKD are now well defined. In ~86% of affected European families, a gene defect localized to 16p13.3 was responsible for ADPKD, while a second locus has been recently localized to 4q13-q23 as candidate for the disease in the remaining families. We present confirmation of linkage to microsatellite markers on chromosome 4q in eight Spanish families with ADPKD, in which the disease was not linked to 16p13.3. By linkage analysis with marker D4S423, a maximum lod score of 9.03 at a recombination fraction of .00 was obtained. Multipoint linkage analysis, as well as a study of recombinant haplotypes, placed the PKD2 locus between D4S1542 and D4S1563, thereby defining a genetic interval of ~1 cM. The refined map will serve as a genetic framework for additional genetic and physical mapping of the region and will improve the accuracy of presymptomatic diagnosis of PKD2.

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common hereditary diseases, with an estimated prevalence of 1:1,000 in Caucasian populations (Dalgaard 1957). Although this is a systemic disease with renal and extrarenal manifestations, it is characterized by the formation of cysts in the kidneys, which can finally lead to complete renal failure. The disease accounts for 9% of patients with end-stage renal disease (ESRD) in Spain (Valles and García-García 1989).

One locus for ADPKD was found to be on the short arm of chromosome 16, within the band p13.3 (Reeders et al. 1985), and the availability of several markers proximal and distal to this locus (PKD1) has improved the genetic diagnosis of the disease by linkage analysis (Breuning et al. 1990; Harris et al. 1991; Peral et al. 1994). The PKD1 gene has very recently been identified (European Polycystic Kidney Disease Consortium 1994). Linkage between the disease and markers close to PKD1 has been excluded in several large ADPKD families, thus establishing evidence for genetic heterogeneity (Kimberling et al. 1988; Romeo et al. 1988; Brissenden et al. 1991; Fossdal et al. 1993; Peral et al. 1993). In a large collaborative study of 328 European ADPKD families, it was estimated that in 86% of families a mutation on the locus PKD1 was responsible for ADPKD (Peters and Sandkuijl 1992). There is clinical evidence that non-PKD1 ADPKD is a milder disease than PKD1 ADPKD, with later onset of cystic disease, hypertension, and ESRD (Parfrey et al. 1990; Bear et al. 1992; Gabow et al. 1992; Ravine et al. 1992).

Recently, two different groups have found evidence of linkage between this second form of the disease and markers on the long arm of chromosome 4, in the region 4q13-q23. Kimberling et al. (1993) have localized this second locus for ADPKD (PKD2) to an interval of ~9 cM, flanked by the markers D4S231 and D4S414, by studying a large family. Peters et al. (1993) demonstrated linkage between the disease and a region flanked by the markers D4S231 and D4S423 in eight non-PKD1 families. Both studies gave sufficient evidence for linkage of ADPKD type 2 to these markers on 4q, with multipoint lod scores (Z) of 12.75 and 22.42, respectively.

Here we present the results of a study set out to confirm the linkage of the disease to the candidate region for PKD2 on 4q in eight Spanish families in which the disease was unlinked to 16p13.3. In addition, we have used microsatellite markers that map within this region, in an attempt to reduce the candidate interval for PKD2.

Families, Material, and Methods

Families and Clinical Data

Eight ADPKD families, with at least four affected individuals available for analysis and in which clear evidence

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against linkage to chromosome 16 markers close to PKD1 has been obtained ($Z < -2$), were selected in a screening of the Spanish population (Peral et al. 1993; authors' unpublished results). Asymptomatic at-risk individuals were examined by ultrasonography; diagnostic criteria were those reported by Bear et al. (1984). No individual <15 years of age is included in the results of the present report. This study was approved by the clinical trials committee of the Ramón y Cajal Hospital.

Laboratory Methods

Genomic DNA was prepared from peripheral blood lymphocytes, as described elsewhere (Peral et al. 1993). PCR was carried out in a 25-μl volume, using *Taq* DNA polymerase and a Perkin-Elmer 480 thermocycler, as described elsewhere (Harris et al. 1991). The $MgCl_2$ concentration and the annealing temperature used for amplification of the microsatellites were 1.5 mM and 57°C, except for D4S1534 (1.0 mM and 55°C) and D4S1563 (1.5 mM and 62°C). The primer sequences used to amplify the newly described microsatellites were as follows: for D4S1563, 5′-GCTGCCTGACACACTGG-3′ and 5′-ACTATTGCTGTTGCTGACCC-3′; and, for AFM353tc1, 5′-AGCTCATATAGGTGTNCTATTCA-3′ and 5′-GTGGGCCTGTCCTGTT-3′. Analysis of 50 independent individuals from CEPH families with these markers revealed a total of 10 alleles for D4S1563 (heterozygosity .64) and five alleles for AFM353tc1 (heterozygosity .57). Fragments were separated on nondenaturing acrylamide gels, and the bands were visualized by staining with ethidium bromide. Bands were scored according to allele sizes.

Genetic Markers and Linkage Analysis

The genetic order and recombination intervals for the microsatellite markers used in this study are presented in figure 1. The map for markers D4S1538, D4S1534, D4S1542, D4S1544, D4S414, and D4S423 has been published previously (Gyapay et al. 1994), but the order for D4S1544 and D4S414/D4S423 could not be resolved with odds >1,000:1. Mapping of the new markers, D4S1563 and AFM353tc1, was accomplished by using the CMAP and CILINK options of the LINKAGE program, after genotyping individuals from eight CEPH families with them. This map fits well with the results obtained after studying 10 Spanish families. Moreover, we have observed a recombination event that confirms the location of D4S1544 proximal to D4S414/D4S423. The location of marker D4S231 (Mills et al. 1992) proximal to D4S1534 is based on our results. Linkage analysis was performed on a Digital DEC 5000 station using LINKAGE program version 5.1 (Lathrop and Lalouel 1984). Two-point lod score and multipoint analyses were done with the MLINK and LINKMAP options of the LINKAGE package. Because of computational limitations in multipoint analysis, the disease locus was tested against only four marker loci at one time. For unaffected at-risk individuals, age-dependent penetrance was taken into account via three liability classes, with penetrances of .5, .85, and .95 for age groups of 15–20 years, 20–30 years, and >30 years, respectively (Fenton and Sandkuijl 1992). The use of other liability classes (as in Kimberling et al. 1993) did not essentially affect our results. For PKD2 a gene frequency of .0001 was assumed (Peters et al. 1993). The allele frequencies for DNA markers in our population were calculated from all available unrelated individuals. Testing for locus heterogeneity was carried out with the HOMOG program version 2.70 (Ott 1991).

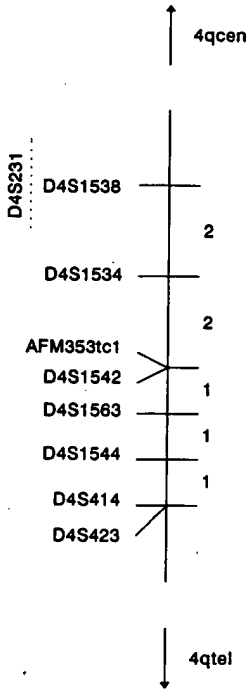

Figure 1 Genetic linkage map of the microsatellite markers used in this study, indicating sex-averaged genetic distance (in cM). The map is based on linkage studies as indicated in Families, Material, and Methods. The location of D4S231 proximal to D4S1534 is based on our results. Marker AFM353tc1 was assayed only in two families having a crossover in this region.

Results

The locus PKD2 has been previously localized to a region of 7–9 cM on chromosome 4q, a region flanked by the proximal marker D4S231 and the distal markers D4S414 and D4S423. We tested eight Spanish non-16p ADPKD families with eight microsatellite markers, including D4S414 and D4S423, from the candidate region on chromosome 4. Figure 1 shows the genetic order and the relative distances between adjacent markers.

The cumulative two-point linkage results are summarized in table 1. The highest Z value was observed between ADPKD and D4S423 ($Z = 9.03$ at $\theta = .00$). For markers

Table 1

Pairwise Z Values for Linkage between ADPKD and Chromosome 4 Markers

Marker	No. of Alleles	Heterozygosity	Z_{max}	Recombination Fraction at Z_{max}
D4S231	7	.71	4.28	.058
D4S1538	5	.70	6.02	.050
D4S1534	6	.77	5.68	.052
D4S1542	3	.45	3.19	.045
D4S1563	10	.64	4.75	.033
D4S1544	5	.61	.87	.000
D4S414	13	.89	5.40	.031
D4S423	9	.83	9.03	.000

D4S1538, D4S231, D4S1534, D4S1542, D4S1563, and D4S414, we also obtained significant Z values. In contrast, the Z value for D4S1544 was nonsignificant, because this marker was not informative in most of the families.

A multipoint analysis was carried out with the most informative markers, to determine which of them flank the PKD2 gene. Because D4S414 and D4S423 showed no recombination, either in the literature (Kimberling et al. 1993; Gyapay et al. 1994) or in our sample, they were collapsed into a single haplotype for multipoint analysis. We first used the subset of markers D4S1534, D4S1542, D4S1563, and D4S414/D4S423. The multipoint analysis yielded a peak Z value (Z_{max}) of 11.10, placing ADPKD most likely between markers D4S1542 and D4S1563 (fig. 2). The difference between the Z_{max} values for the placement of PKD2 within this interval is >3.0, when compared with all the other possible locations proximal to D4S414/D4S423, and is >2.0 for locations distal to D4S414/D4S423, thus suggesting that the placement of PKD2 between D4S1542 and D4S1563 is correct (Ott 1991). A similar result was obtained when marker D4S1538, instead of D4S1534, was included in the multipoint analysis (data not shown). When analyzed individually via multipoint analysis, each family yielded evidence of linkage to this area, with Z_{max} values of 0.88–2.40. Heterogeneity analysis with the HOMOG test provided no evidence of genetic heterogeneity (the estimated proportion of linked families, α, was 1.00, with 95% confidence interval .65–1.00).

In three families, interesting recombinants were observed in chromosome 4 haplotypes. In family 6030 (fig. 3), an identical haplotype was observed to cosegregate

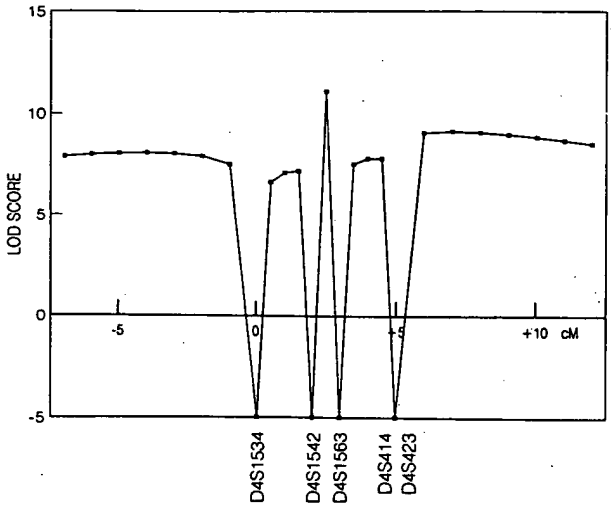

Figure 2 Multipoint linkage analysis between PKD2 and chromosome 4 markers, for eight families, performed using LINKMAP program. D4S1534 was arbitrarily assigned position 0, and the other loci were positioned at their corresponding genetic distance (in cM): D4S1542 at +2 cM, D4S1563 at +3 cM, and D4S414 and D4S423, which were collapsed into a single locus, at +5 cM.

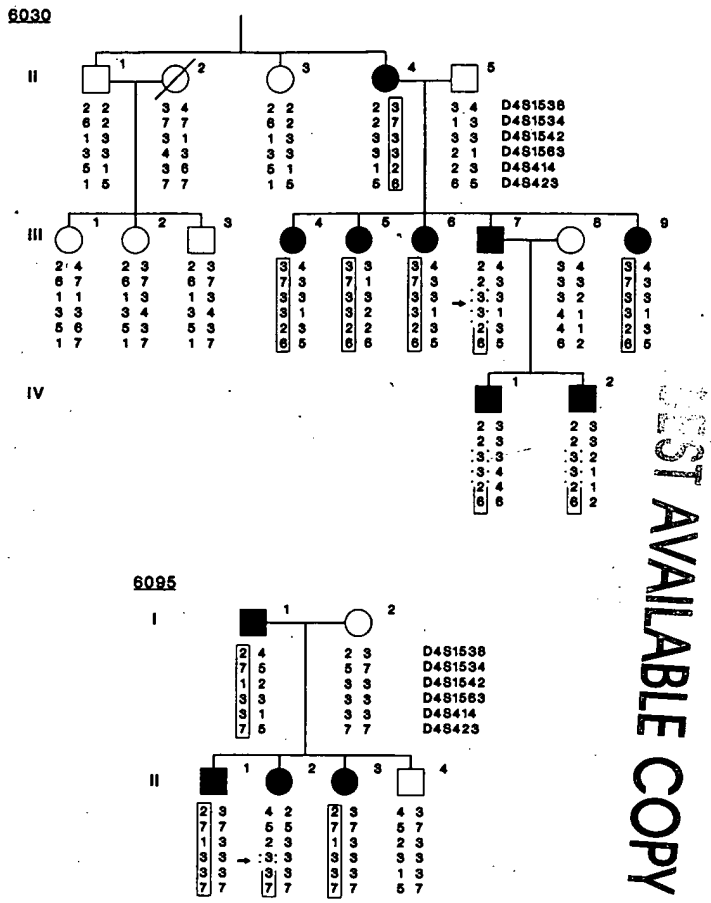

Figure 3 Genotypes at six markers on chromosome 4q for families 6030 and 6095. Alleles for individual II-2 (6030) were deduced from his offspring. Phase for individual III-8 (6030) could not be determined, since one of her two children carried a recombinant haplotype. Alleles from the original nonrecombinant haplotype that cosegregated with the disease in each family are boxed (dashed lines indicate that those markers were not informative in the corresponding parental meiosis). The arrows indicate recombination events that localize the PKD2 locus most likely distal from D4S1542. Genotypes for markers linked to PKD1 (16p) and to PKD2 (4q), in the eight families, can be obtained from the authors on request.

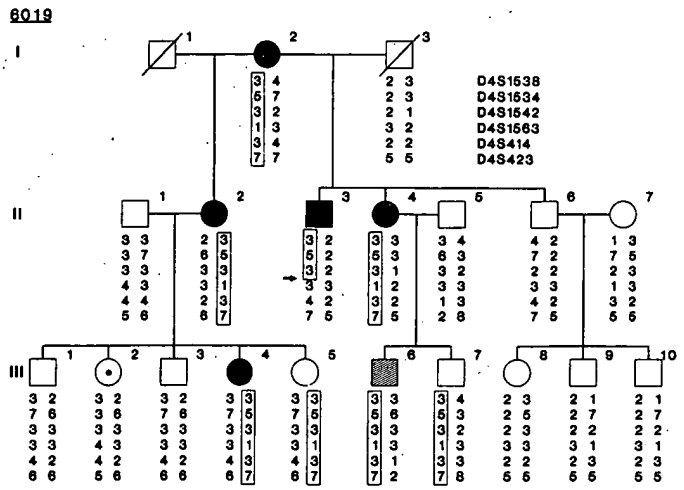

Figure 4 Genotypes at six markers on chromosome 4q for family 6019. Alleles for individual I-3 were deduced from his offspring. Individual III-6 (21 years old; represented as a crosshatched square) has two cysts in one kidney and is carrying the affected haplotype in this family. Individual III-2 (32 years old; represented as a dotted circle) has a solitary cyst in one kidney, and we have considered this fact without relation to the disease. The arrow indicates a recombinant that localizes the PKD2 locus most likely proximal from D4S1563. Other symbols are as in fig. 3.

with the ADPKD phenotype in the progeny of the only affected sib in the second generation (II-4). This haplotype is evident in patients III-4, III-5, III-6, and III-9, but it is not present in the apparently unaffected individuals II-1 and II-3, who were last examined at ages 70 and 66 years, respectively. The affected individual III-7 shares with his affected sibs his affected mothers's (II-4) alleles at D4S414 and D4S423, but he has received different alleles at D4S1538 and D4S1534. Since markers D4S1542, D4S1563, and AFM353tc1 (this last one is not shown in fig. 3) were not informative, this recombination positioned the disease gene distal to D4S1534. The two other affected individuals (IV-1 and IV-2) have received the new disease-bearing haplotype from their father (III-7).

In family 6095 (fig. 3), individual II-2 shares with her affected sibs (II-1 and II-3) her affected father's (II-1) alleles at D4S414 and D4S423, but she has inherited different alleles at D4S1538, D4S1534, and D4S1542, as has her healthy brother (II-4). This recombination positioned the PKD2 locus distal to D4S1542. Markers D4S1563 and D4S1544 (not shown) were not informative.

In family 6019 (fig. 4), an identical haplotype was observed to cosegregate with the disease, since it appears in four affected members of three generations (I-2, II-2, II-4, and III-4), but it is not present in the healthy individuals II-6, III-1, and III-3, who are 54, 37, and 35 years old, respectively. This haplotype was also found in three asymptomatic subjects: III-5 (17 years old), III-7 (19 years old), and III-6 (21 years old). This last subject already presents two cysts in one kidney. A solitary cyst was found in individual III-2, but, taking into account her age (32 years) and the fact that her affected sister (III-4, who is only 1 year older) presents several cysts in both kidneys, hepatic cysts, and some degree of renal impairment, we considered that subject III-2 has not inherited the disease. The existence of these four individuals with an uncertain ultrasonographic scan raises the possibility that the disease is not linked to PKD2 in this family. However, we think that the reported latter expression of the disease in the non-PKD1 cases, as well as the existence of individuals with solitary renal cysts in the normal population (as would be the case for individual III-2), would explain this apparent nonpenetrance. The affected individual II-3 presents a recombinant haplotype that arose in his mother by a crossover between markers D4S1542 and D4S1563. The recombinant chromosome carries alleles for markers D4S1538, D4S231, D4S1534, and D4S1542 from the affected chromosome, as well as the alleles for markers D4S1563 and D4S414 from the normal chromosome. Markers AFM353tc1 (not shown), D4S1544 (not shown), and D4S423 were not informative. This recombination positions the disease centromeric to marker D4S1563. The analysis of recombinant families was therefore consistent with the multipoint data, and the most likely position for PKD2 was between D4S1542, on the proximal side, and D4S1563, on the distal side.

Discussion

In this study we have confirmed the linkage between polymorphic markers of chromosome 4q13-q23 and ADPKD in eight Spanish families previously shown to be unlinked to the PKD1 locus on chromosome 16. Multipoint analysis supports the linkage of the disease to this area, with a Z_{max} of 11.10. No evidence of further locus heterogeneity was found in our sample.

Previous genetic studies have shown that the second locus for ADPKD (PKD2) lies in a 7–9-cM region bounded by the markers D4S231 (on the proximal side) and D4S414/D4S423 (on the distal side). An important prerequisite for physical mapping of a disease gene is the identification of flanking markers closely linked to it. Flanking markers can be defined by the demonstration of recombination between such markers and the disease locus. We have used microsatellite polymorphic markers that map within this region with the following order: D4S1538/D4S231-(.02)-D4S1534-(.02)-AFM353tc1/D4S1542-(.01)-D4S1563-(.01)-D4S1544-(.01)-D4S414/D4S423. The order between D4S231 and D4S1538 could not be determined. Recombinants in three of the families tentatively localize the PKD2 gene between D4S1542 and D4S1563, in a 1-cM interval, and multipoint analyses give sufficient support for placing the disease locus in this area.

This study reinforces the idea that a gene on chromosome 4q is responsible for most if not all ADPKD not linked to 16p, although analysis of more families is re-

quired before we can reach a definite conclusion. Using the markers presented in this study, we are now in position to determine if any other non-PKD1 phenotypes are linked to this region and to determine if there is further genetic heterogeneity within this group of disorders. Several authors have suggested a milder ADPKD phenotype for the second form of the disease, since a later appearance of ultrasonographically detectable cysts, as well as later onset of ESRD, were observed in those families (Bear et al. 1992; Gabow et al. 1992; Ravine et al. 1992). We found a similar pattern in the families presented in the present study. While the mean age at onset of ESRD was 54.2 (±8.1) years for persons with the PKD1 form of the disease, in the five cases of ESRD among the PKD2 families the mean age at onset was 66.2 (±3.3) years. In these families, two persons 20–30 years of age have inherited the disease-bearing haplotype but do not have renal cysts. No comparison with the PKD1 families was possible, because of the low number of individuals. These facts have to be taken into account when we establish the penetrance of the second form of the disease. Since no data from penetrance of PKD2 are available yet, we used liability classes as in PKD1 (Fenton and Sandkuijl 1992). However, we confirmed that other liability classes do not dramatically affect the final result.

The results of this study have refined the candidate region for the PKD2 locus on chromosome 4q. This information will be useful for presymptomatic diagnosis of the second form of the disease. As has been the case for other disease genes, recombinant families and new polymorphisms will be very important to further narrow this interval, and in our laboratory work is in progress to isolate novel microsatellites from this area.

Acknowledgments

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